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Resonance energy transfer study of hemoglobin and cytochrome *c* complexes with lipids

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Abstract

The complexes of hemoglobin and cytochrome *c* with liposomes composed of phosphatidylcholine and its mixtures with cardiolipin and cholesterol have been studied by monitoring resonance energy transfer between fluorescent probe 3-methoxybenzanthrone as donor and heme groups of the proteins as acceptors. By analyzing experimental data within the framework of the model of energy transfer in two-dimensional systems, the limits of the range of possible heme positions with respect to lipid bilayer have been assessed. The distance of heme group of hemoglobin from the membrane center was found to increase in the presence of cardiolipin or cholesterol. The results obtained for cytochrome *c* complexes with cardiolipin-containing model membranes suggest the existence of preferential protein orientation relative to the lipid bilayer, and provide evidence for the protein penetration in the membrane interior.

Keywords: Fluorescence energy transfer; Hemoglobin; Cytochrome *c*; Protein–lipid complex

1. Introduction

Resonance energy transfer (RET) is presently widely used for obtaining information on the structure of biological macromolecules and their assemblies [1–3]. In particular, this technique proved to be relevant to structural characterization of the protein–lipid complexes, being a major constituent of biomembranes [4–7]. Despite the extensive studies of protein–lipid interactions, a number of important aspects of the problem remain to be clarified. One of such aspects concerns the factors responsible for the character of protein disposition in the lipid bilayer. In ascertaining these factors it seems of great interest to examine protein–lipid complexes by studying RET between fluorophores bound to lipid and protein molecules.

In the present investigation an attempt has been made to apply this technique for elucidating the structure of model protein–lipid systems. A protein component of the systems under study was represented by hemoglobin or cytochrome *c*, while a lipid one included liposomes composed of phosphatidylcholine (PC) and its mixtures with cardiolipin (CL) and cholesterol. These systems were chosen to study for the following reasons. PC is a zwitterionic phospholipid, being the main component of the most biological membranes. Negatively charged phospholipid CL occurs in large amounts in mitochondrial and bacterial membranes. Cholesterol (Chol) is a neutral lipid, abundant in a lot of membranes, that is known as effective modulator of the lipid bilayer fluidity [8]. Hemoglobin (Hb) is not a membrane protein; however, as demonstrated in a number of studies, it can

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bind to lipids forming complexes stabilized by electrostatic and hydrophobic interactions [9–15]. Unlike Hb, cytochrome *c* (cyt *c*) is a peripheral membrane protein, occurring in the inner mitochondrial membrane. Binding of cyt *c* to lipid bilayer strongly depends on the presence of negatively charged phospholipids since the protein has significant positive charge (ca. 8–9) at physiological pH [16–20]. Given all these factors, investigation of the model systems chosen may be of importance not only in gaining insight into the general features of protein–lipid interactions, but also in the further understanding the mechanisms governing cyt *c* association with the mitochondrial membrane. The present study was focused on the examination of Hb and cyt *c* complexes with lipids by monitoring RET between fluorescent probe 3-methoxybenzanthrone (MBA) localized in the lipid phase and heme groups of the proteins. The main question addressed concerns the effect of lipid composition of the model membranes on the heme position with respect to the lipid bilayer.

2. Materials and methods

2.1. Chemicals

Egg yolk PC and beef heart CL were purchased from Bakpreparat (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform/methanol/acetic acid/water (25:15:4:2, v/v). Bovine heart cyt *c* was obtained from Reakhim (Russia). MBA was from Zonde (Latvia). Horse hemoglobin and thiourea were purchased from Reanal (Hungary).

2.2. Preparation of liposomes

A stock suspension of unilamellar phospholipid vesicles was prepared by the method of Batzri and Korn [21]. One ml of the ethanol lipid solution containing appropriate amounts of PC, CL and cholesterol was injected into 13 ml of 5 mM sodium phosphate buffer (pH 7.4) under continuous stirring. Ethanol was then removed by dialysis. Phospholipid concentration was determined according to the procedure of Bartlett [22].

2.3. Protein binding studies

Quantitative characteristics of Hb and cyt *c* binding to liposomes were determined by analyzing lipid-induced changes of the heme absorbance in Soret band. It was assumed that the observed absorbance decrease (ΔA_{407}) is proportional to the concentration of bound protein (B):

$$\Delta A_{407} = aB \tag{1}$$

where a is a coefficient of proportionality. If the protein binding site contains n lipid molecules, total number of such sites (N_0) is equal to L_o/n and association constant (K_b) is given by:

$$K_b = \frac{B}{F(N_o - B)} \tag{2}$$

where L_o is a total lipid concentration, F is the concentration of free protein. Eq. 2 can be written as:

$$F = \frac{B}{K_b(N_o - B)} \tag{3}$$

From Eqs. 1 and 3, one obtains the following expression for the total protein concentration (P_o):

$$P_o = B + F = \Delta A_{407}\left(\frac{1}{a} + \frac{n}{K_b(L_o a - n\Delta A_{407})}\right) \tag{4}$$

Eq. 4 was used in non-linear regression analysis aimed at obtaining the values of K_b, n and a. Calculated values of P_o were compared with experimental ones, determined spectrophotometrically. Initial estimates of binding parameters were derived from the double reciprocal plots, using the following approximations. If $P_o > B$, Eq. 4 can be written as:

$$\frac{1}{\Delta A_{407}} = \frac{n}{K_b L_o a P_o} + \frac{n}{L_o a} \tag{5}$$

The x-intercept of the plot of $1/\Delta A_{407}$ *vs.* $1/P_o$ equals K_b, while the y-intercept gives a value of $n/L_o a$. By varying the lipid concentration and considering the case when $L_o/n > B$ one obtains:

$$\frac{1}{\Delta A_{407}} = \frac{n}{K_b L_o a P_o} + \frac{1}{P_o a} \tag{6}$$

The y-intercept of the plot of $1/\Delta A_{407}$ vs. $1/L_o$ equals $1/P_o a$, thus giving approximate value of a. The estimates of a were derived from the results of protein titration by liposomes.

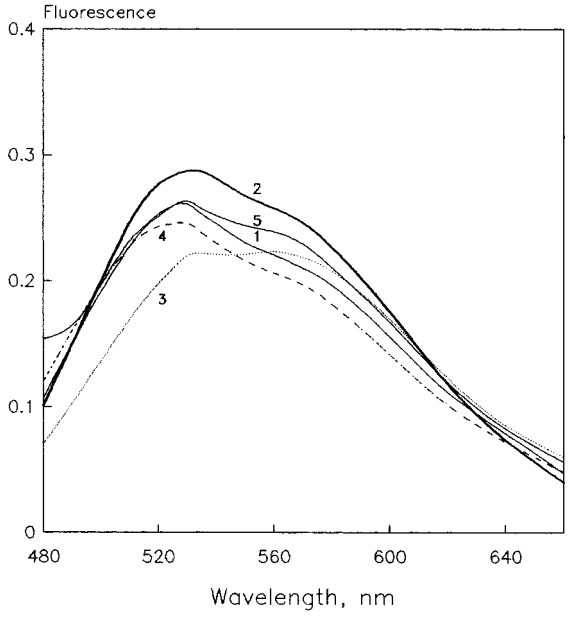

Fig. 1. Fluorescence spectra of MBA in the liposomal suspensions: 1, PC; 2, PC:CL (9:1, mol/mol); 3, PC:CL (1:1); 4, PC:Chol (4:1); 5, PC:Chol:CL (7:2:1).

Hb and cyt *c* were used in the oxidized state, characterizing by relatively high stability in the presence of liposomes [11–15]. Protein concentrations were found using extinction coefficients $E_{407} = 5.66 \cdot 10^5$ $M^{-1}\,cm^{-1}$ for Hb [23] and $E_{407} = 1.05 \cdot 10^5$ $M^{-1}\,cm^{-1}$ for cyt *c* [24].

2.4. Fluorescence measurements

Fluorescence measurements were performed with F-1040 spectrofluorimeter (Hitachi, Japan). Emission spectra of MBA were excited at 440 nm. Excitation and emission slit widths were set at 5 nm. Non-linear least-squares fitting of MBA emission spectra to a sum of Gaussian curves was performed using commercially available software Peakfit (Jandel Scientific).

Fluorescence intensity measured in the presence of Hb or cyt *c* was corrected for reabsorption and inner filter effects using the following coefficients [1,25]:

$$k = \frac{\left(1-10^{-A}\right)A_s}{\left(1-10^{-A_s}\right)A} \tag{7}$$

where A is the absorbance of MBA in the absence of the protein, A_s is the total absorbance of the sample at excitation or emission wavelengths. Quantum yields of MBA in liposomal suspensions were estimated using fluorescein solution as standard [26]. Critical distance of energy transfer (R_o, nm) was calculated as [1]:

$$R_o = 979\left(K^2 n_r^{-4} Q_d J\right)^{1/6} \tag{8}$$

where J is the overlap integral, n_r is the refractive index of the medium ($n_r = 1.37$), K^2 is an orientation factor ($K^2 = 2/3$), Q_D is the donor quantum yield. For donor–acceptor pair MBA–heme of Hb, R_o values were found to be 3.7 nm (PC liposomes), 3.7 nm (PC:CL (9:1)), 3.9 nm (PC:CL (1:1)), 3.8 nm (PC:Chol (4:1)), 3.9 nm (PC:Chol:CL (7:2:1)). For pair MBA–heme of cyt *c* in all cases studied (CL-containing liposomes), R_o was determined to be 3.8 nm. Concentration of MBA employed in RET experiments was 5.9 μM. To avoid protein-induced lipid peroxidation, antioxidant thiourea was added to the liposomal suspensions at a concentration of 100 mM.

2.5. Theory

To analyze RET in membrane systems, a number of theoretical models have been proposed [27–32].

Table 1
Distinct components of MBA fluorescence spectra in liposomal suspensions, resolved by Peakfit

Liposomes	Component 1		Component 2	
	λ_{max} (nm)	%	λ_{max} (nm)	%
PC	554	90	518	10
PC:CL (9:1)	556	91	518	9
PC:CL (1:1)	563	94	521	6
PC:Chol (4:1)	549	91	515	9
PC:Chol:CL (7:2:1)	545	9	522	2

Experimental data obtained in the present study were interpreted quantitatively in terms of the modified model of Wolber and Hudson [28], developed for the case of donors and acceptors randomly distributed in a plane. The main statements of this model can be briefly described as follows. By denoting the lifetime of an excited donor in the absence and presence of acceptors τ_d and τ_{da}, respectively, the rate of de-excitation of the donor fluorescence is given by:

$$k = \tau_{da}^{-1} = \tau_d^{-1}\left(1 + \sum_{i=1}^{N}(R_o/R_i)^6\right) \quad (9)$$

where R_i is the distance between donor and ith acceptor, N is the number of acceptors within the disc of radius R_d, beyond which energy transfer is insignificant. If the concentration of acceptors per unit area is denoted by C_a^s, N, is given by:

$$N = \pi R_d^2 C_a^s \quad (10)$$

Since de-excitation of donor fluorescence exhibits first-order kinetics the following relationships hold:

$$-\frac{dP(t)}{dt} = kP(t) = -\tau_d^{-1}\left(1 + \sum_{i=1}^{N}(R_o/R_i)^6\right)P(t) \quad (11)$$

$$P(t) = \exp(-t/\tau_d)\prod_{i=1}^{N}\exp\left[(-t/\tau_d)(R_o/R_i)^6\right] \quad (12)$$

where $P(t)$ is the probability that donor excited at time $t=0$, is still excited at time t, $P(0)=1$. If donor is surrounded by N acceptor molecules the ensemble average decay $P_N(t)$ is expressed by:

$$P_N(t) = \exp(-t/\tau_d)\prod_{i=1}^{N}\int_{R_s}^{R_d} c\exp\left[(-t/\tau_d)(R_o/R_i)^6\right]W(R_i)dR_i \quad (13)$$

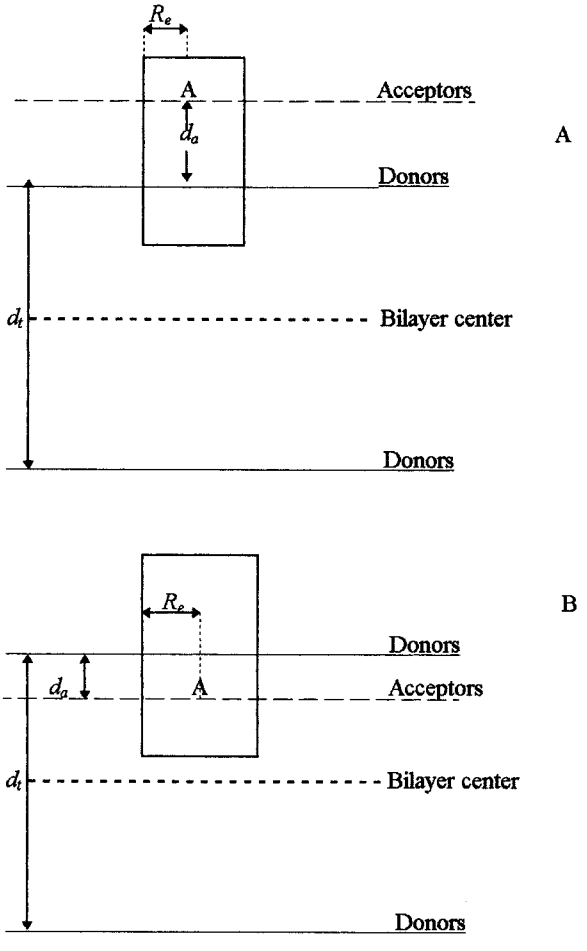

Fig. 2. Possible modes of the localization of donor and acceptor planar arrays in the lipid bilayer.

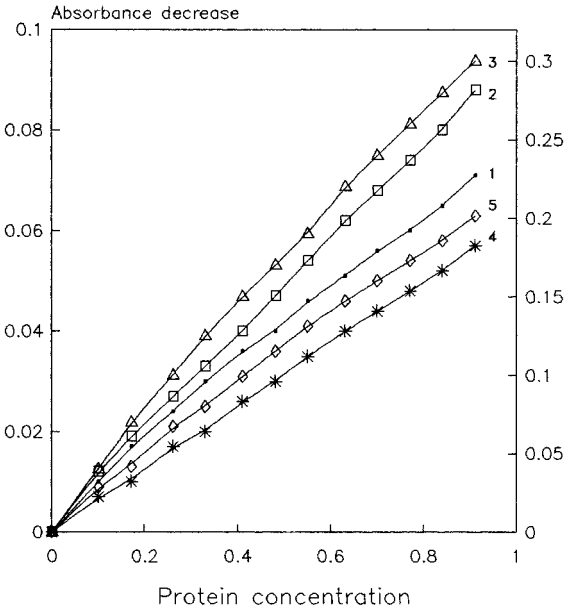

Fig. 3. Lipid-induced decrease of Hb absorbance at 407 nm as a function of the protein concentration (μM): 1, PC, lipid concentration $C_L = 0.4$ mM; 2, PC:CL (9:1), $C_L = 0.39$ mM; 3, PC:CL (1:1), $C_L = 0.22$ mM; 4, PC:Chol (4:1), $C_L = 0.55$ mM; 5, PC:Chol:CL (7:2:1), $C_L = 0.43$ mM. The right y-axis corresponds to curve 3.

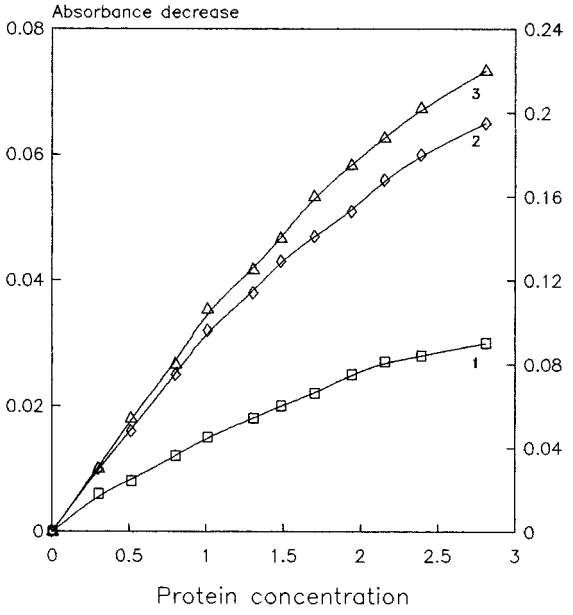

Fig. 4. Decrease of cyt *c* absorbance at 407 nm in the presence of liposomes: 1, PC:CL (9:1), $C_L = 0.39$ mM; 2, PC:CL (1:1), $C_L = 0.22$ mM; 3, PC:Chol:CL (7:2:1), $C_L = 0.43$ mM. The right *y*-axis corresponds to curve 2, protein concentration is given in μM.

where $W(R_i)\mathrm{d}R_i$ is the probability of finding *i*th acceptor in the annulus between radii R_i and $R_i+\mathrm{d}R_i$. Relative quantum yield of a donor (Q_r) can be written as:

$$\frac{Q_{DA}}{Q_D} = \frac{\int_0^\infty P_N(t)\mathrm{d}t}{\int_0^\infty \exp(-t/\tau_d)\mathrm{d}t} = \int_0^\infty \exp[-\lambda](I(t))^N \mathrm{d}\lambda \tag{14}$$

$$I(t) = \int_{R_s}^{R_d} \exp\left[-\lambda(R_o/R)^6\right] W(R)\mathrm{d}R \tag{15}$$

where Q_D, Q_{DA} are donor quantum yields in the absence and presence of acceptors, respectively, $\lambda = -t/\tau_d$. A random distribution of donors is characterized by identical $W(R_i)$ for all values of *i*, so that one can write $W(R_i) = W(R)$. In general case $W(R)$ is given by:

$$W(R) = \frac{2R\mathrm{d}R}{R_d^2 - R_e^2 - d^2} \tag{16}$$

where R_e is the distance of closest approach between donor and acceptor, *d* is a vertical separation of donor and acceptor planar arrays in the membrane.

3. Results

The aforementioned approach provides a theoretical basis for analysis of RET in the model protein–lipid systems studied. To apply this approach, surface concentration of bound acceptor must be known as well as some assumptions on the donor localization in the lipid bilayer are needed. In view of this the main steps of the present study include: (i) assessment of the donor localization in the model membranes; (ii) determination of the binding parameters characterizing the complexes of Hb and cyt *c* with liposomes and subsequent evaluation of C_a^s; (iii) measurements of relative quantum yield of the donors at various acceptor concentrations and analysis of experimental data.

3.1. Localization of donors in lipid bilayer

The first set of experiments was aimed at examining the donor binding to liposomes and estimation of their quantum yields. To date, the properties of fluorescent probe, used in this work as donor, are rather

Table 2
Parameters of hemoglobin and cytochrome *c* binding to liposomes

Liposomes	Hemoglobin			Cytochrome *c*		
	K_b (10^5 M^{-1})	*n*	*a* ($\times 10^5$)	K_b (10^5 M^{-1})	*n*	*a* ($\times 10^4$)
PC	4.3 ± 1.1	310 ± 44	4.4 ± 0.7			
PC:CL (9:1)	3.5 ± 0.8	236 ± 40	5.0 ± 0.7	4.5 ± 1.3	180 ± 37	5.6 ± 0.9
PC:CL (1:1)	4.1 ± 1.0	127 ± 28	15 ± 2.0	6.3 ± 1.2	50 ± 16	20 ± 3
PC:Chol (4:1)	1.5 ± 0.5	220 ± 36	4.4 ± 0.8			
PC:CL:Chol (7:2:1)	3.2 ± 0.8	330 ± 48	4.8 ± 0.7	8.3 ± 1.3	108 ± 22	5.6 ± 0.8

well characterized. MBA is a neutral hydrophobic probe which is localized in the glycerol backbone region of the lipid bilayer, distributing between the outer and inner leaflets [26,33]. Quantum yield of MBA in the suspension of liposomes is a factor of ca. 40 greater then that in buffer solution, so that possible contribution of the free probe in measured fluorescence appears to be negligible. The MBA Q_D values obtained in our experiments do not differ significantly for liposomes of various composition and are found to be ca. 0.1. Analysis of fluorescence spectra of MBA in liposomal suspensions (Fig. 1) via Peakfit revealed two distinct spectral components, reflecting the existence of the two main populations of membrane-bound probe. An emission maximum of the predominant longer wavelength component varied from 545 to 563 nm for different liposomes and exhibited shift to a shorter wavelength in the presence of cholesterol (Table 1). This finding is consistent with those reported elsewhere [26], and is attributed to decreased polarity of the probe's microenvironment, arising from the displacement of hydrational water by cholesterol. Contribution of the minor short wavelength component to the overall

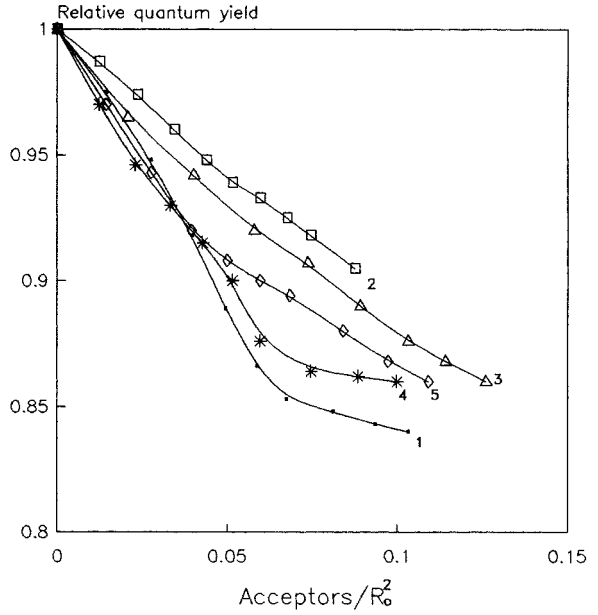

Fig. 5. Relative quantum yield of MBA vs. concentration of heme groups of membrane-bound Hb (given as a quantity (number of acceptors)/R_o^2): 1, PC, $C_L = 0.47$ mM; 2, PC:CL (9:1), $C_L = 0.77$ mM; 3, PC:CL (1:1), $C_L = 0.43$ mM; 4, PC:Chol (4:1), $C_L = 0.41$ mM; 5, PC:Chol:CL (7:2:1), $C_L = 0.63$ mM.

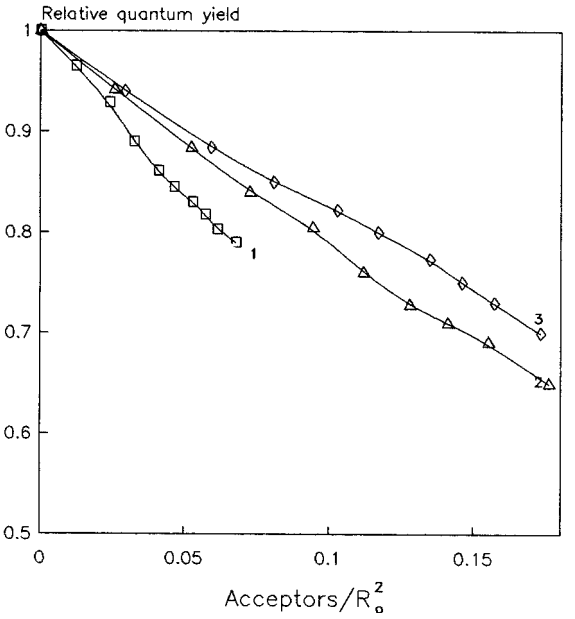

Fig. 6. Relative quantum yield of MBA as a function of the concentration of heme groups of cyt *c*, associated with liposomes: 1, PC:CL (9:1), $C_L = 0.77$ mM; 2, PC:CL (1:1), $C_L = 0.43$ mM; 3, PC:Chol:CL (7:2:1), $C_L = 0.63$ mM.

fluorescence spectrum was found not to exceed 10%. Since emission wavelength maximum is influenced by various factors, including, in particular, polarity of the probe's surroundings and it's rotational mobility, it is difficult to ascertain unambiguously whether the existence of two populations of MBA can be a consequence of the differences in the depth of probe penetration in the membrane interior. Nevertheless, taking into account that only one component considerably contribute to the overall fluorescence spectrum, it seems reasonable to assume that in the lipid monolayer donors are distributed in a plane. Thus, in analyzing the results of RET measurements one should consider two planes of donors, located in the outer and inner leaflets. If the acceptors are assumed to be situated on the outer side of the membrane and separated from a nearest plane of donors by a distance d_a, expression for $W(R)$ would be analogous to Eq. 16:

$$W_1(R) = \frac{2RdR}{R_d^2 - R_e^2 - d_a^2} \quad (17)$$

Fig. 2 illustrates the different possibilities of acceptor localization with respect to donor planes. For donors, situated at the inner bilayer leaflet, the follow-

ing relationships hold (Fig. 2A and B, respectively):

$$W_2(R) = \frac{2RdR}{R_d^2 - R_e^2 - (d_t + d_a)^2} \quad (18)$$

$$W_2(R) = \frac{2RdR}{R_d^2 - R_e^2 - (d_t - d_a)^2} \quad (19)$$

where d_t is the distance separating donor planes. By assuming uniform distribution of the donors between two planes, relative quantum yield can be represented as follows:

$$Q_r = 0.5\left(\int_0^\infty \exp\left[-\lambda\right](I_1(t))^N d\lambda + \int_0^\infty \exp\left[-\lambda\right](I_2(t))^N d\lambda\right) \quad (20)$$

where

$$I_1(t) = \int_{R_s}^{R_d} \exp\left[-\lambda(R_o/R)^6\right] W_1(R)dR \quad (21)$$

$$I_2(t) = \int_{R_s}^{R_d} \exp\left[-\lambda(R_o/R)^6\right] W_2(R)dR \quad (22)$$

It is noteworthy that the case of acceptor localization on both sides of membranes can be treated in analogous manner.

3.2. Binding of Hb and cyt c to liposomes

The formation of protein–lipid complexes was examined by monitoring the absorption spectra of Hb and cyt *c* in visible region. Association of the proteins with liposomes results in the decrease of the heme absorbance in Soret band. This effect was observed in a number of studies, and is usually explained by the ability of Hb and cyt *c* to induce lipid peroxidation, leading, in turn, to substantial alterations in the protein's structure [12–15,34]. Since the critical distance of RET is crucially dependent on the spectral overlap of donor and acceptor, any change in the heme absorption spectrum may affect the donor fluorescence, thus preventing reliable interpretation of the data. To avoid such ambiguities all RET measurements were performed in the presence of antioxidant (thiourea), while samples used in the binding studies did not contain any antioxidant added.

In Figs. 3 and 4, the absorbance changes of Hb and cyt *c* observed upon addition of liposomes are plotted vs. total protein concentration. Analysis of these data in terms of the aforementioned approach (Eqs. 1–6) allowed to estimate binding parameters characterizing the protein–lipid complexes under study (Table 2). It should be pointed out that the changes in the absorbance of cyt *c* were observed only in the presence of negatively charged liposomes (i.e., liposomes, containing CL), although there exists some evidence suggesting the possibility of cyt *c* interaction with neutral bilayers [35–38]. Furthermore, correction coefficients calculated from Eq. 7 for the system PC–cyt *c* did not differ noticeably from the empirical ones, determined as a ratio of the donor fluorescence intensities measured in the absence and presence of the protein. This observation may be considered as confirming the relevance of Eq. 7 to correction for inner filter and reabsorption effects. The values of K_b and n, presented in Table 2, were used for calculation of the amount of bound protein according to the relationship, derived from the solving of Eq. 2:

$$B = 0.5\left[P_o + L_o/n + 1/K_b - \sqrt{(P_o + L_o/n + 1/K_b)^2 - 4P_oL_o/n}\right] \quad (23)$$

The surface acceptor concentration was determined as follows:

$$C_a^s = \frac{N_A B}{S_L} \quad (24)$$

$$S_L = N_A L_o (f_{PC}S_{PC} + f_{CL}S_{CL} + f_{CH}S_{CH}) \quad (25)$$

where N_A is Avogadro number, f_{PC}, f_{CL}, f_{CH} are mole fractions of PC, CL and cholesterol, respectively, S_{PC}, S_{CL}, S_{CH} are mean areas per lipid molecule. In the absence of cholesterol S_{PC} and S_{CL} were taken to be 0.65 nm^2 [8] and 1.2 nm^2 [39], respectively. Given the condensing effect of cholesterol [40], for model membranes, containing this lipid, the following values were used: $S_{PC} = 0.50$ nm^2, $S_{CL} = 1.0$ nm^2, $S_{CH} = 0.39$ nm^2. To obtain the concentration of bound acceptor in units, natural for two-dimensional

systems (i.e., a number of acceptors per R_o^2) the C_a^s values were multiplied by R_o^2.

3.3. Resonance energy transfer studies

Shown in Figs. 5 and 6 are the plots of the relative quantum yield of MBA vs. concentration of the bound heme of Hb or cyt *c*. The main purpose of the further data analysis was to obtain the sets of parameters R_e, d_a, d_t, providing the best fit of experimental Q_r values to Eq. 20. The fitting of Q_r calculated by numerical integration of Eq. 20 (Q_r^t), to that determined experimentally (Q_r^e) was made by minimizing the following function:

$$f = \frac{1}{n_a}\sum_{i=1}^{n_a}(Q_r^e - Q_r^t)^2 \quad (26)$$

where n_a is the number of acceptor concentrations employed in RET measurements.

Since MBA is localized presumably at the boundary between hydrophilic and hydrophobic regions of the lipid bilayer [26,33], the chosen values of d_t were close to the thickness of hydrocarbon core, being ca. 2.0–2.4 nm [8]. Parameter R_e, corresponding to the minimum lateral donor–acceptor distance (i.e., distance between the centers of the probe and heme), equals the sum of the donor radius (r_d), taken to be 0.5 nm [26], and the distance of heme center from the protein surface (r_h). Note that experimental data can be fitted with the similar accuracy by numerous sets of parameters R_e, d_a, d_t. However, by considering the real size of membrane and protein, some limitations can be introduced. Molecule of Hb is known to consist of four subunits forming spheroid with dimensions $6.4\times5\times5$ nm and heme groups being localized in the vicinity of the protein surface. In RET experiments protein concentrations fall in the range 0.17–1.7 μM, where dissociation of Hb into dimers is known to occur [9]. This change in the quaternary structure of Hb, followed by the exposure of non-polar face, is thought to be one of the reasons for substantially greater Hb propensity to hydrophobic interactions with lipids, as compared with other water soluble proteins [9,10]. If one assumes that Hb binds to liposomes in the form of dimer or separate subunits with average radius ca. 1.3 nm, a reasonable choice for R_e limits appears to be 1.3–3 nm.

Molecule of cyt *c* is a globule with dimensions $3\times3.4\times3.4$ nm [41]. Heme is located in the center

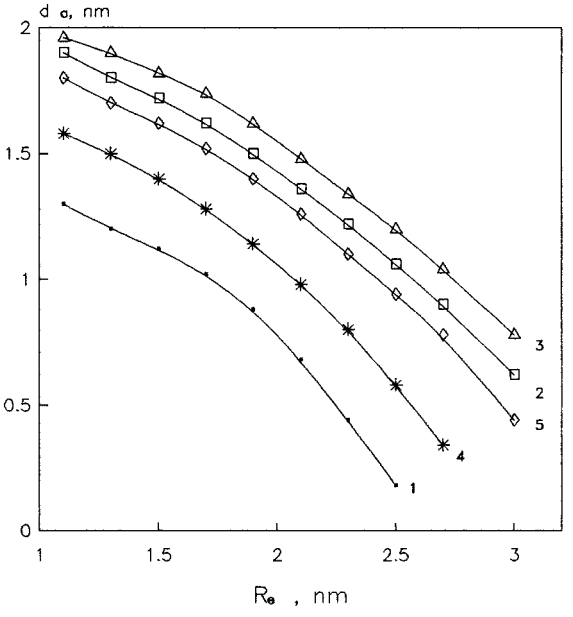

Fig. 7. Relationship between parameters d_a and R_e, derived for donor–acceptor pair MBA–heme of Hb from the fitting of experimental data to Eq. 20, Eq. 21, and Eq. 18 (with d_t being equal to 2 nm). 1, PC; 2, PC:CL (9:1); 3, PC:CL (1:1); 4, PC:Chol (4:1); 5, PC:Chol:CL (7:2:1).

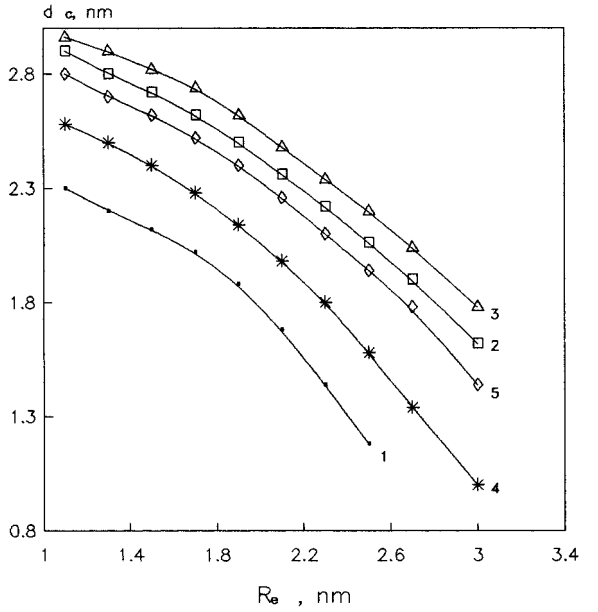

Fig. 8. The distance of Hb heme from the lipid bilayer center (d_c) plotted vs. R_e: 1, PC; 2, PC:CL (9:1); 3, PC:CL (1:1); 4, PC:Chol (4:1); 5, PC:Chol:CL (7:2:1).

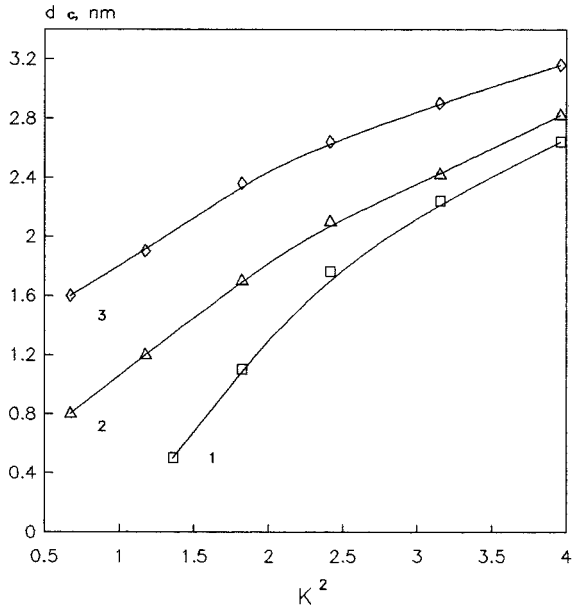

Fig. 9. Dependencies of d_c on K^2 derived for donor–acceptor pair MBA–heme of cyt *c*: 1, PC:CL (9:1); 2, PC:CL (1:1); 3, PC:Chol:CL (7:2:1).

of the protein, about 1.5 nm from its surface. Supposing that cyt *c* can change its conformation upon binding to lipids and taking heme radius to be ca. 0.6 nm, it seems probable that R_e values can range from 1.1 to 4.0 nm.

4. Discussion

Fig. 7 illustrates typical relationships between d_a and R_e, obtained at a given d_t for donor–acceptor pair MBA–heme of Hb, assuming that acceptors are localized at the outer side of membrane. On the basis of such data, the distance between heme and bilayer center (d_c) can be determined as follows:

$$d_c = d_a + 0.5d_t \quad (27)$$

Presented in Fig. 8 are d_c values calculated according to Eq. 27. It is noteworthy that upon varying d_t in the range 2.0–2.8 nm alterations in d_c do not exceed 0.2 nm. Although it seems impossible to settle the most probable value of R_e, some tendencies in d_c behavior can be pointed out. Inclusion of CL in PC bilayer leads to increase of the heme distance from the membrane midplane. Similar in sign, but less in magnitude changes of d_c occur when 20 mol% of cholesterol is added to PC. There are at least two possible explanations for this effect, involving (i) decrease of the depth of protein penetration into lipid bilayer in the presence of CL or cholesterol, and (ii) structural alterations in Hb molecule accompanied by the change of heme position with respect to the contact region in the protein–lipid complex. Under experimental conditions employed in the present study, net surface charge of Hb appears to be about zero, since the isoelectric point of this protein is ca. 7.4. In view of this it seems likely that interaction of Hb with lipids is predominantly hydrophobic. Numerous data available in the literature confirm Hb ability to interact with the non-polar part of the membrane. Such observations as increase of liposome ion permeability caused by Hb [9,42], the similarities between Hb and integral membrane proteins in their effect on the lipid thermotropic behavior [43], and Hb ability to associate with zwitterionic lipids at high ionic strength [9] are interpreted in terms of the protein penetration in the hydrophobic region of lipid bilayer. Total hydrophobicity of Hb does not differ significantly from that of other soluble proteins [10], but the peculiar structure

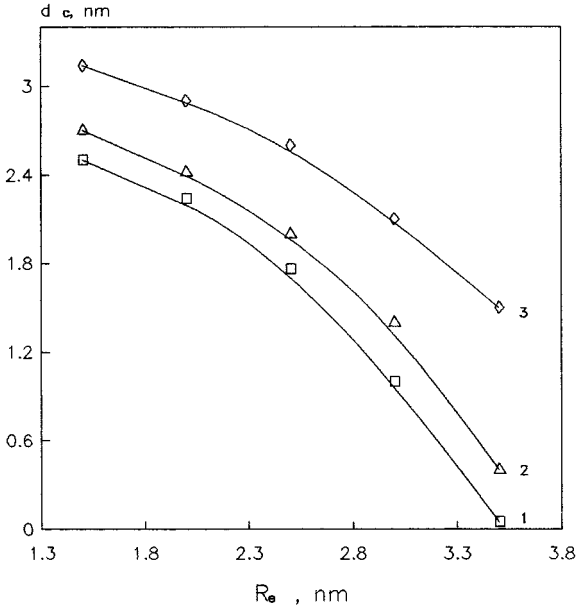

Fig. 10. The distance of cyt *c* heme from the lipid bilayer center as a function of R_e: 1, PC:CL (9:1); 2, PC:CL (1:1); 3, PC:Chol:CL (7:2:1).

of this protein and the possibility of its dissociation into dimers or separate subunits enhances Hb affinity for non-polar part of the membrane. As follows from the results of the present work, in complex of Hb with PC liposomes the distance between heme and bilayer center falls in the range 1.2–2.3 nm. The increase of this distance, observed upon addition of cholesterol, presumably reflects the less depth of the protein penetration in the membrane interior due to decreased motional freedom of the lipid acyl chains induced by cholesterol [44]. In model membranes, containing CL, formation of electrostatic contacts between positively charged protein side groups and phosphate groups of CL can, to some extent, prevent penetration of the protein in lipid bilayer. Furthermore, one cannot rule out the possibility of CL-induced conformational changes of the protein, since negatively charged phospholipids are reported to be capable of substantially altering the structure of Hb [12–15]. It should also be mentioned that in lipid bilayers consisting of CL or its mixtures with other phospholipids, addition of the proteins can result in the formation of non-bilayer structures, particularly hexagonal H_{II} phase [45]. By analyzing information obtained to date, we could not find any arguments in favor of appearance of H_{II} phase in the systems containing CL and Hb. However, there exist ^{31}P-NMR data suggesting the formation of non-bilayer structures in model membranes composed of PC and phosphatidylethanolamine [46]. It is assumed that such structures can facilitate the protein penetration through the membrane [45]. In view of these findings we have tried to analyze experimental results supposing that acceptors (Hb heme groups) are localized at both sides of the lipid bilayer. In this case satisfactory fitting of the data was achieved only for liposomes, comprising PC and CL. The heme distance from the membrane center was found to be ca. 1.0–2.2 nm. Assuming that heme resides in the part of Hb subunit opposite to segment, penetrating in the membrane and taking subunit diameter to be ca. 2.5 nm, one obtains that these d_c estimates correspond to practically full insertion of the protein in the lipid bilayer. Thus, the results of the present study do not exclude, in principle, the possibility of Hb translocation through liposomal membranes composed of PC and CL, perhaps due to formation of non-bilayer structures.

It is important to emphasize that all results discussed above were obtained supposing that the coefficient K^2 in Eq. 8 is equal to 0.67, the value corresponding to a random reorientation of the donor emission and acceptor absorption moments during the donor emission lifetime. This assumption is generally thought not to introduce considerable error in the data analysis, since R_o depends on the one-sixth power of K^2, donors and acceptors usually possess some rotational mobility, and multiple electronic transitions appear to be responsible for donor fluorescence [28–30]. However, if there exist certain preferable orientations of the donor and acceptor dipoles, the K^2 value may substantially differ from 0.67. Note that K^2 can change from 0 to 4; the minimum value corresponds to perpendicularly oriented donor and acceptor dipoles, while the maximum one characterizes the case when these dipoles are parallel and identically directed [1]. Relative orientation of the donor and acceptor dipoles is difficult to determine experimentally, but uncertainty in K^2 may lead to considerable ambiguities in the data interpretation. The existence of a specific lipid-binding site on the protein molecule would result in particular heme orientation with respect to the lipid bilayer. The possible contribution of orientation factor in RET efficiency is discussed in a number of studies [27–30,47–55]. Detailed analysis of the role of this factor in determining energy transfer between membrane-bound fluorescent probes (*n*-(9-anthroyloxy) stearic acids) and heme of Hb has been performed in the works of Eisinger et al. [48–50]. The range of possible K^2 values was found to be rather small (with an average K^2 of 0.5), even if Hb should have specific binding sites for lipids [50]. It was shown that uncertainty in the distance of heme acceptors from the donor plane, introduced by orientational effects, does not exceed 20% [48].

In a series of comprehensive studies by Gryczynski et al., an approach has been developed that allowed to calculate orientation parameter for the case of energy transfer between tryptophan and heme in hemoglobin and myoglobin [51–55]. This approach is based on the use of spectroscopic data on the orientation of transition moments relative to the planes of indole or porphyrin rings in combination with X-ray structural information on the position of tryptophan residues and hemes in the protein molecule.

Unfortunately, because of the complexity of the system examined in the present work, any accurate assessment of the K^2 bounds for donor–acceptor pair MBA–heme of Hb appears to be impossible. This is caused, first of all, by the lack of information on the orientation of MBA dipole in the lipid bilayer. Anisotropy of MBA fluorescence in the model membranes was measured to be ca. 0.05 [33], with orientational distribution of the probe characterized by a half-angle of ca. 47°. This finding provides evidence for noticeable rotational mobility of MBA in the lipid bilayer. Information on heme orientation within Hb molecule can, in principle, be derived from X-ray studies, but these data cannot be used directly in analyzing the results of solution experiments. Furthermore, heme position may be affected by the lipid-induced conformational changes of the protein. In particular, there exist some indications that Hb interaction with phospholipids can lead to the loosening of the bonds between heme and globin [12–15]. On the other hand, as follows from the estimates of Eisinger [50], symmetrical heme disposition in Hb molecule results in the compensation of the contributions of various heme groups to orientation factor, leading to an average K^2 value of 0.5. It should also be pointed out that the use of orientation factor corresponding to a random distribution of donors and acceptors yields rather reasonable values of the heme distance from the bilayer center (Fig. 8). Taken together, all these facts suggest that the K^2 value employed in the data analysis (0.67) is not far from a true one.

Meanwhile, the existence of preferential heme orientation may be one of the reasons for complications occurring in interpreting the results obtained for donor–acceptor pair MBA–heme of cyt *c*. In this case all attempts to fit experimental quenching profiles of MBA (Fig. 6) putting K^2 equal to 0.67 proved to be unsuccessful. As can be seen in Figs. 5 and 6, cyt *c* causes more drastic decrease of Q_r compared with Hb, reflecting more pronouncing ability of cyt *c* to quench MBA fluorescence. One of the possible explanations for this effect could involve the increased contribution of orientation factor in RET efficiency due to specific position of cyt *c* molecule with respect to the lipid bilayer. This assumption is supported by some observations reported in the literature. Using K^2 as one of the parameters in the data fitting, Teissie concluded that cyt *c* has specific orientation in the complexes with monolayers and vesicles prepared from PC and phosphatidic acid, with heme crevice being fully accessible to aqueous phase [17]. In this context it is relevant to note that cyt *c* is known to have a peculiar structure, characterizing by segregation of acidic and basic groups on the protein surface into two positively charged clusters with a negative patch between them [41]. In the present work we examined the complexes of cyt *c* with negatively charged CL-containing liposomes. Taking into account that there exist two clusters of positively charged residues on the surface of cyt *c*, one cannot exclude the possibility of preferential orientation of this protein relative to the charged model membranes being studied. Given all these factors, we have tried to analyze experimental data (Fig. 6), assuming that K^2 can vary from 0.67 to 4. As indicated above, heme is localized in the center of cyt *c*, a distance of ca. 1.5 nm from its surface [41]. Provided that the size of the protein molecule and heme position are not influenced significantly by lipids, the most probable R_e value would be equal to the sum of donor radius (0.5 nm) and heme distance from the protein surface (1.5 nm), i.e., 2 nm. Presented in Fig. 9 are the sets (K^2, d_c), giving satisfactory fits of experimental curves to those predicted by the model, assuming that acceptors are distributed at the outer side of liposomal membrane and putting R_e equal to 2 nm. The distance of heme from the membrane center (d_c) appeared to increase upon increasing the content of CL in the bilayer and addition of cholesterol (Fig. 9), the effects being similar to those observed for Hb. However, it should be noted that a number of studies provide arguments in favor of lipid-induced alterations in cyt *c* structure [36,38,55–58]. In particular, as revealed by deuterium NMR, cyt *c* can adopt loosened or 'unfolded' conformation upon interaction with CL [56,57]. Taking into account the possibility of the protein structural changes, we have analyzed the experimental data supposing that R_e can vary in the limits 1.5–3.5 nm (for a given K^2). The character of the dependencies $d_c(R_e)$ obtained in such a way is illustrated in Fig. 10. Note that maximum d_c, corresponding to the case when $R_e = 1.5$ nm and $K^2 = 4$, was estimated to be ca. 3.5 nm. Denoting the thickness of the lipid bilayer by d_m and protein radius by R_P, the depth of

the protein penetration in the bilayer (d_P) can be determined as:

$$d_P = 0.5d_m - (d_c - R_P) \quad (28)$$

Taking d_m to be ca. 4.6 nm [8] and R_P to be 1.5 nm, it follows that for d_c varying from 0.1 to 3.5 nm, d_P values fall in the range 0.3–3.7 nm, and the lowest d_P are derived for liposomes, containing cholesterol. These results are consistent with the findings of other authors, suggesting that cyt *c* can penetrate in the bilayer [35–38]. In particular, evidence for cyt *c* insertion in the interior of model membranes consisting of dioleoylphosphatidylglycerol has been obtained in the calorimetric studies of Zhang and Rowe [36]. Using the Raman spectroscopy technique to examine cyt *c* association with dipalmitoylphosphatidylcholine, Vincent and Levin have shown that the protein can interact with hydrocarbon chains of the lipids [35]. All these notwithstanding, one should bear in mind that in CL-containing systems, studied in the present work cyt *c* can specifically alter lipid organization, inducing the formation of non-bilayer structures such as inverted micelles and hexagonal H_{II} phase [16]. As pointed out in an excellent review of de Kruijff et al. [45], the formation of H_{II} phase in the presence of cyt *c* suggests that the protein resides within the aqueous cylinders spanning the bilayer. Besides, a certain fraction of the bound protein is supposed to form lipid-soluble complexes with CL (inverted micelles) accounting for cyt *c* ability to permeate through the bilayer [45]. It seems also noteworthy that cholesterol can promote the formation of H_{II} phase [59]. Given all these facts, in analyzing the data we consider the possibility of acceptor localization on both sides of the membrane. However, in this case we failed to achieve a satisfactory data fit yielding reasonable d_c values. The aforementioned estimates of the depth of cyt *c* penetration in the membrane do not exclude, in principle, the possibility of the full protein insertion in the bilayer that is thought to occur upon formation of H_{II} phase or inverted micelles. Indeed, thermodynamically favorable deep penetration of positively charged cyt *c* into the lipid bilayer seems to be hardly probable without involvement of non-bilayer structures, promoting this process.

In conclusion, the results of the present study can be summarized as follows. Efficiency of resonance energy transfer between MBA and heme of Hb is found to depend on the lipid composition of model membranes. In analyzing experimental data, the heme position with respect to donor plane was defined by the two parameters reflecting the distance of closest approach of donor and acceptor (R_e) and vertical separation of the donor and acceptor planes (d_a). By varying R_e in the range consistent with the protein dimensions, the limits of the heme distance from the lipid bilayer center (d_c) were ascertained to be ca. 1.0–2.9 nm. This distance increases by a factor of ca. 1.3–1.7 upon inclusion of 10 mol% or 50 mol% of CL into PC bilayers. Similar in sign, but less in magnitude changes of d_c values are caused by cholesterol.

Resonance energy transfer studies performed with the donor–acceptor pair MBA–heme of cyt *c* provide evidence for existence of preferential protein orientation with respect to the lipid bilayer. Assessment of the range of possible heme positions suggests the penetration of cyt *c* in the membrane interior. The depth of the protein insertion in the lipid bilayer was found to decrease upon increasing CL content or inclusion of cholesterol in the liposomal membranes.

The results obtained do not rule out the possibility of protein-induced formation of non-bilayer structures in the model systems containing CL.

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